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TITLE: Quantification of Protein Signatures in Archived Human Prostate Tissues Using Shotgun Proteomic Methods

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14. ABSTRACT

Biomarkers that robustly predict the metastatic potential of localized CaP are sorely needed to effectively treat localized CaP patients that pose the greatest risk of developing significant CaP. Biomarkers specific to significant CaP are also necessary if more effective drugs are going to be developed that can target and cure patients afflicted by this deadly disease. Proteins represent some of the most powerful molecular biomarkers to human disease such as cancer. Therefore this proposal will implement state-of-the-art methods in biological mass spectrometry to identify protein biomarkers specific to non-significant and significant CaP. These new protein biomarkers may spur the development of molecular tests that robustly predict the metastatic potential of non-significant CaP. These tests would reduce the physical and mental burdens associated with the overtreatment of patients afflicted by localized CaP. Also, protein biomarkers specific to significant CaP may represent new and effective drug targets to cure patients already afflicted by this deadly disease. We anticipate this proposal will identify the critical molecular targets with the greatest potential to improve the treatment and potentially cure CaP in men.

15. SUBJECT TERMS
None provided.

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INTRODUCTION

Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

My laboratory is currently constructing protein expression libraries of matched normal and cancerous human prostate tissues using quantitative mass spectrometry. Our research progress over the past 12 months has entailed the processing of archived frozen tissues of normal, T2, and T3 staged prostate tissues for proteomic analyses using label-free, quantitative mass spectrometry. We have acquired tissue blocks of whole prostate radial prostatectomies and have focused on optimizing protocols to extract and profile proteins in matched normal and diseased tissue samples using directed mass spectrometry methods. The ultimate outcome of these efforts will be the identification of pathologically-staged protein biomarkers to organ-confined and metastatic human prostate cancers.

BODY

This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Provide data explaining the relationship of the most recent findings with that of previously reported findings. Appended publications and/or presentations may be substituted for detailed descriptions of methodology but must be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work must be approved by the Army Contracting Officer Representative. This approval must be obtained prior to initiating any change to the original Statement of Work.

Task: *Specific aim1: Construction of mass spectrometry-based protein expression libraries of normal prostate tissue, non-significant CaP tissue, and significant CaP tissue.*

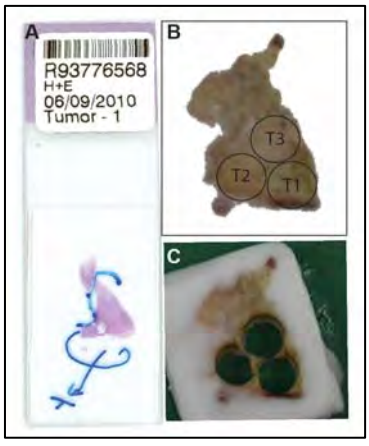

Figure 1. Frozen tissue sample block and hematoxylin and eosin (H&E) stained slide.
A) Pathologist scored H&E slide of paired tumor and adjacent normal prostate tissue sample.
B) Tumor prostate tissue sample with marked 5 mm diameter cores.
C) Photograph of cored tumor prostate tissue sample.

Over the past year my laboratory has acquired 5 frozen radical prostatectomy (RP) samples containing T2 stage prostate cancer and normal adjacent tissue to build protein expression signatures of non-significant (organ-confined) and significant (metastatic) human prostate cancer using label-free, quantitative mass spectrometry. Last year we were able to extract up to 100 micrograms of total protein using whole-mount FFPE RP tissue block samples. This was a significant improvement in the amount

of extractable protein that was isolated in needle-biopsied tiss ue cores in the first year of this research application (*e.g.* 1-5 ug of total protein). We have been able to increase the amount of extracted protein up to 1 mg of N-linked glycoproteins using frozen whole-mount tissue blocks (**Fig. 1**). This has greatly enhanced our ability to in terrogate the protein expression patterns in non-significant and significant human prostate cancers by nanospray liquid-chromatography tandem mass spectrometry (nano-LC-MS/MS). The frozen tissue blocks provide up to 500X more material to identify human prostate cancer protein biomarkers.

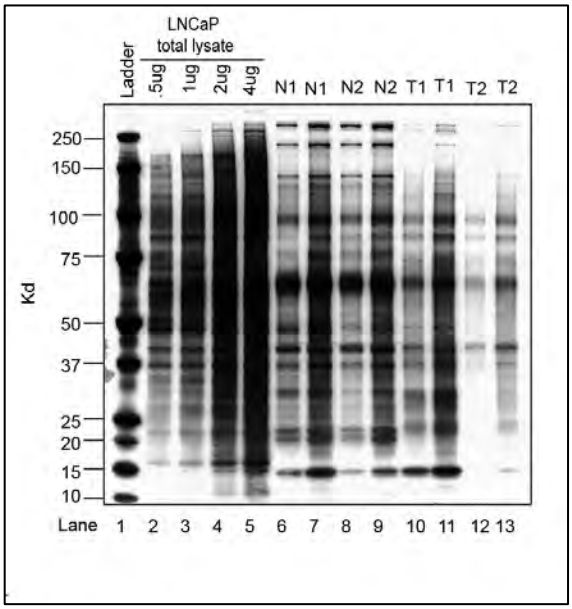

Figure 2. Silver-stained SDS-PAGE gel of total protein extracted from sample R90197381. Detergent solubilized proteins were run into lanes 6-13. A protein standard composed of LNCaP whole cell lysates at .5ug, 1ug, 2ug, and 4ug was loaded into lanes 2-5. Protein isolated from normal adjacent tissues, samples 1 and 2, were loaded into lanes 6-9 (sample 1, lanes 6-7; sample 2, lanes 8-9). Protein isolated from tumor tissues, samples 1 and 2, were loaded into lanes 10-13 (sample 1, lanes 10-11; sample 2, lanes 12-13). 1 (lanes 6, 8, 10, and 12) and 2 (lanes 7, 9, 11, and 13) microliters of sample were subjected to SDS-PAGE.

My laboratory has also optimized a new protein-extraction protocol to interrogate glycoproteins in frozen tissue samples using lectin-affinity chromatography methods. This glycoprotein enrichment protocol facilitates the extraction of N-lin ked glycoproteins in tissue and facilitates the p roteomic interrogation of low-abundance biomarkers in tissue samples. Briefly, tissue samples were extracted with detergent and processed by SDS-PAGE to determine the quantity and quality of tissue extracted proteins (**Fig. 2**). The detergent-solubiliz ed proteins were subsequently incubated with wheat-germ agglutinin (WGA) and con canavalin-A (ConA) beads, washed, and el uted with sol uble n-acetyl glucosamine. The elut ed glycoproteins were dialyzed to remove residual detergent, and both the integrity and quantity of glycosylated protein samples was determined by SDS-PAG E and silver-stain analysis (**Fig. 3**). Equal amounts of tumor and normal adjacent sample wer e digested with trypsin and subjected to strong cation exchange chromatography (**Fig. 4**). The individual peptide fractions were collected, desalted, and subjected to nano-LC-MS/MS. This protocol facilitates the mass spectrometry analysis of glycoprot eins in c ancerous lesions of the prostate and adjacent normal tissue. We have preliminary MS data s howing the differentiall y expression of glyc oproteins such as prostate-specific antige n (PSA) and prostatic specific phosphatase (PSAP), which are well-known androgen-regulated glycoproteins and biomarkers used in the d etection of localized prostate cancers (1).

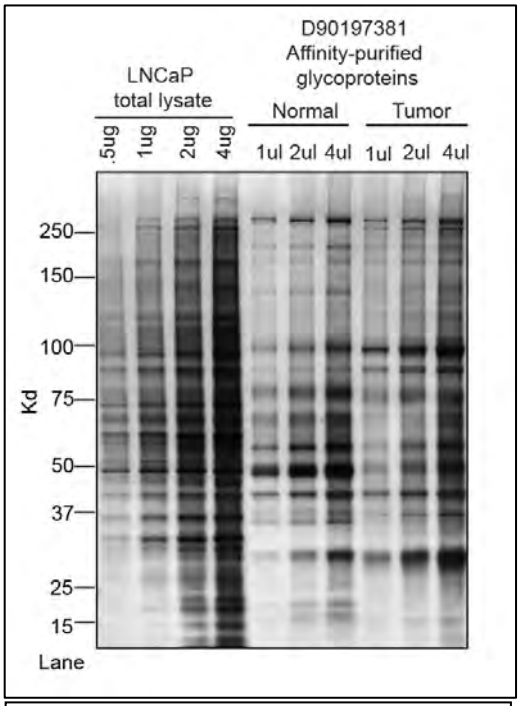

Figure 3. Silver stain of affinity-purified glycoproteins from tumor and normal adjacent prostate tissue (sample D90197381).

To expedite the discovery of pathologically-staged protein

biomarkers to non-significant and significant human prostate cancer in archived tissue samples, last year my laboratory implemented a new mass spectrometry workflow called "Directed Proteomics"(2). In contrast to shotgun proteomic methods, which attempts to sequence all ionized tryptic peptides in a complex samples using data-dependent (DD) acquisition methods, we have instead opted for a mass spectrometry profiling strategy to detect and sequence peptide ion differences across multiple tissue samples. This directed proteomic approach have been superior to our past shotgun proteomic experiments. This reflects our ability to sequence lower intensity peptide ions in the complex tissue sample relatively to shotgun proteomic protocols that routinely selects high-abundant peptide ions for MS/MS. We are in the process of submitting a manuscript detailing the strengths of this new data-acquisition scheme to identify and quantify protein biomarkers in archived tissue samples shortly.

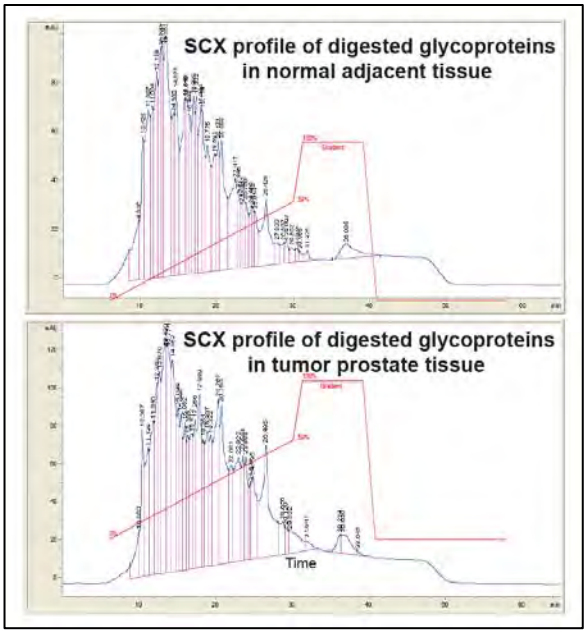

Figure 4: U.V. trace chromatogram of strong cation exchanged (SCX) fractionated peptides of affinity-purified glycoprotein tissue samples of paired normal and tumor tissue samples. Each sample was subjected to a 65 minute gradient of 0-59% buffer B (300 mM KCl) and peptides were collected every 1 min for 40 min for a total of 40 SCX fractions.

Last year, my laboratory established a new collaboration with Dr. Michael B. Cohen, the departmental head of Pathology at the UI Carver College School of Medicine. We have continued our collaboration with Dr. Cohen over the past year, as his laboratory has provided pathological expertise in the annotation of archived frozen radical prostatectomy tissue blocks. Dr. Cohen's has volunteered his time to help my laboratory carefully annotate the Gleason score of non- significant and significant human prostate cancer samples.

We will complete the processing of cancerous and normal tissue samples over the next year, as these samples will allow us to build comprehensive protein expression libraries of normal prostate tissue, non-significant prostate cancer tissue, and significant prostate cancer tissue.

Task: *Specific aim 2: Identification of protein biomarkers in non-significant and significant CaP tissues using novel statistical methods.*

My laboratory has also been working with LabKey, the developers of Computational Portal Analysis System (CPAS) database, to develop plug-in statistical modules to identify statistically significant protein biomarkers of non-significant and significant human prostate cancer samples.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

• My laboratory is generating glycoprotein expression profiles of normal prostate tissue, non-significant prostate cancer tissue, and significant prostate cancer tissue by targeted proteomic methods.

• We have developed a glycoprotein tissue-extraction protocol has been developed for the proteomic analysis of archived frozen tissue samples.

• We have successfully implemented a directed proteomic approach that has significantly improved our ability to identify and quantify protein expression changes across normal prostate tissue, non-significant prostate cancer, and significant human prostate cancer.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

My laboratory is actively profiling tissue samples at the moment. However, our preliminary analyses have identified glycoprotein expression differences between cancerous and adjacent normal tissue. These differentially expressed glycoproteins encode regulatory enzymes (*e.g.* DNA-dependent protein kinase-DNAPKC, entonucleotide triphosphate diphosphohydrolase 5-ENTPD5) that have been implicated in the development and invasiveness of localized prostate cancers (3, 4). We will have completed all reported outcomes over the next reporting period.

CONCLUSION: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

The proposed research studies are ongoing. We have not reached any conclusions regarding the completed research to date. We anticipate over the next reporting period that distinct protein expression patterns will be obtained on normal prostate tissue, non-significant prostate cancer, and significant human prostate cancer. These findings will identify novel protein biomarkers to non-significant and significant human prostate cancer.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

1. Tosoian J, Loeb S 2010 PSA and beyond: the past, present, and future of investigative biomarkers for prostate cancer. ScientificWorldJournal 10:1919-1931
2. Schmidt A, Claassen M, Aebersold R 2009 Directed mass spectrometry: towards hypothesis-driven proteomics. Curr Opin Chem Biol 13:510-517
3. Brenner JC, Ateeq B, Li Y, Yocum AK, Cao Q, Asangani IA, Patel S, Wang X, Liang H, Yu J, Palanisamy N, Siddiqui J, Yan W, Cao X, Mehra R, Sabolch A, Basrur V, Lonigro RJ, Yang J, Tomlins SA, Maher CA, Elenitoba-Johnson KS, Hussain M, Navone NM, Pienta KJ, Varambally S, Feng FY, Chinnaiyan AM 2011 Mechanistic rationale for inhibition of poly(ADP-ribose) polymerase in ETS gene fusion-positive prostate cancer. Cancer Cell 19:664-678
4. Fang M, Shen Z, Huang S, Zhao L, Chen S, Mak TW, Wang X 2010 The ER UDPase ENTPD5 promotes protein N-glycosylation, the Warburg effect, and proliferation in the PTEN pathway. Cell 143:711-724

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

None reported to date.

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

Data analyses are not complete. Completed datasets will be provided over the next reporting period.